

The poly(ADP-ribose) polymerase-1 inhibitor 3-aminobenzamide suppresses cell growth and migration, enhancing suppressive effects of cisplatin in osteosarcoma cells

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Received December 28, 2010; Accepted February 18, 2011

DOI: 10.3892/or.2011.1212

Abstract. Pharmacological inhibition of DNA repair pathways has been emerging as an effective tool for cancer treatment. Poly(ADP-ribose) polymerase (PARP) is involved in DNA repair and transcriptional regulation and is now recognized as a key regulator of cell survival and cell death. *In vitro* and *in vivo* data suggest that PARP inhibitors could be used not only as chemo/radiotherapy sensitizers but also as single agents to selectively kill cancer cells in certain types of tumors. In the present study, we investigate the effects of 3-aminobenzamide (3-AB), a potent inhibitor of PARP, on human osteosarcoma cells and whether or not it can sensitize the tumor cells to chemotherapeutic agents. The results indicated that 3-AB suppressed U2OS cell growth in a time- and dose-dependent manner, and the suppressive effects of 3-AB were associated with increased cell apoptosis. In addition, 3-AB suppressed cell invasion *in vitro* and enhanced the suppressive effects of cisplatin in U2OS cells. Our work suggests that this PARP-1 inhibitor may be developed into an effective agent for the treatment of human osteosarcoma.

Introduction

Osteosarcoma is a primary malignant bone tumor with a high tendency to metastasize. Although it can occur at any age, osteosarcoma has a peak incidence in the young adolescents. It often originates in the metaphyses of long bones, such as the distal femur, proximal tibia and proximal humerus (1). Current treatment recommendations for osteosarcoma include neoadjuvant chemotherapy and surgical resection, followed by

postoperative chemotherapy. Chemotherapy protocols involve multiagents and may include doxorubicin, cisplatin, ifosfamide and methotrexate (2). The introduction of multi-agent chemotherapy dramatically improved the outcome, of patients who present with no metastatic disease; ~70% will be long-term survivors. However, a significant number of patients will still relapse. The average time to relapse is 1.6 years and recurrences usually occur as pulmonary metastases or, less frequently, metastases to distant bones or local recurrences (3).

Research in osteosarcoma has been focused on identifying which patients will respond to the current therapy. The goal is to identify the patients whose tumor will resist chemotherapy or whose tumor will metastasize or recur. These patients could potentially be treated differently. The second area of focus has been to identify new targets for therapy that may be more effective or less toxic, especially for the tumors that respond poorly to the conventional agents (4).

Since DNA repair pathways can enable tumor cells to survive DNA damage that is induced by chemotherapeutic treatments, the efficacy of DNA-damaging chemotherapeutic drugs can be modulated by DNA repair pathways. This makes DNA repair mechanisms a promising target for novel cancer treatment (5). The potential to inhibit DNA repair enzymes as a mechanism of improving existing cancer treatments has been the focus of drug development programs worldwide.

Over 40 years ago, poly (ADP-ribose) polymerases (PARPs), a family of highly conserved enzymes found in plants and animals, were first described (6). PARP-1 is the most studied PARP, with a molecular weight of 114 kDa that catalyzes the addition of branched poly (ADP) ribose polymers on itself as well as other acceptor proteins (7). PARP-1 is involved in DNA repair and transcriptional regulation and is now recognized as a key regulator of cell survival and cell death as well as a master component of a number of transcription factors involved in tumor development and inflammation (8). Overexpression of PARP-1 has been reported in various human malignancies, such as malignant lymphoma (9), breast carcinoma (10), Ewing's sarcoma (11), HCC (12) and in the U2OS human osteosarcoma cell line (13). As PARP-1 actively participates in DNA repair, PARP-1 inhibitors have been applied in cancer treatment either alone or in combination with DNA-damaging agents (14). Several studies have demonstrated

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Key words: poly (ADP-ribose) polymerase, 3-aminobenzamide, human osteosarcoma, cisplatin

that pharmacological inhibition or genetic ablation of PARP-1 not only provided remarkable protection from tissue injury in various oxidative stress-related disease models but also resulted in a clear beneficial outcome in the treatment of cancer (15). In certain types of tumors including lung cancer, colon carcinoma and cervical cancer, PARP-1 inhibition was shown to be an effective means of enhancing tumor sensitivity to radiation and chemotherapy (16-19), also PARP inhibitor could be used as single agents to selectively kill cancers defective in DNA repair, specifically cancers with mutations in the breast cancer-associated genes (BRCA1 and BRCA2) (20,21). Additionally, a number of reports have shown a relationship between PARP and angiogenesis, at least five PARP inhibitors have been efficiently used *in vitro* (22-24) to inhibit vascular endothelial growth factor (VEGF)-induced proliferation, migration, and tube formation in human umbilical vein endothelial cells (HUVECs). However, whether or not PARP-1 inhibitor is able to suppress tumor cell growth and migration and whether it improves chemotherapeutic effects in human osteosarcoma have rarely been studied. 3-aminobenzamide (3-AB) is a potent inhibitor of PARP-1, in the present study we investigate the suppressive effects of 3-AB alone and in combination with a chemotherapeutic agent in human osteosarcoma cells.

Materials and methods

Cell line and culture. The human osteosarcoma cell line U2OS was maintained by the orthopedics laboratory, Tongji Hospital, Tongji Medical College. The U2OS cells were cultured with McCoy's 5 α medium modified (Sigma), containing 10% fetal bovine serum supplemented with penicillin (100 U/ml) and streptomycin (100 μ g/ml). The cells were cultured at 37°C in a humidified incubator with 5% CO₂.

Cell proliferation assay. Cells were seeded in three 96-well plates (3000 cells/well), the cells were treated with varying concentrations of 3-AB, after 24, 48 and 72 h, the viability of the cells was assessed by the Cell count kit-8 (Beyotime), the number of viable cells was determined by measuring the absorbance of each well at 450 nm.

Cell apoptosis analysis. Cells were seeded in 6 plates with a 6-cm diameter (5 \times 10⁴ cells/plate). The plates were divided into two groups; cells in one group were cultured with complete growth medium, while in the other group were cultured with complete growth medium plus 3-AB at different concentration. Cells were harvested at 24, 48 and 72 h after incubation. After two washes with cold PBS, the cells were stained for 15 min with annexin V-FITC and propidium iodide in 500 μ l binding buffer, and then analyzed by flow cytometry (Becton-Dickinson Immunocytometry Systems, USA) within 1 h. Data were recorded with a typical annexin V-FITC scatter gram; the annexin V⁺/PI⁺ cell population (right bottom quadrant) represents early apoptosis cells.

Caspase-3 activity assay. The same number of U2OS cells was seeded in 4 plates. One plate was used as the control, the other three plates were treated with 10 mM 3-AB for 8, 12 and 24 h, and then the cells were harvested, the caspase-3 activity was assayed by caspase-3 colorimetric assay kit (Appligen

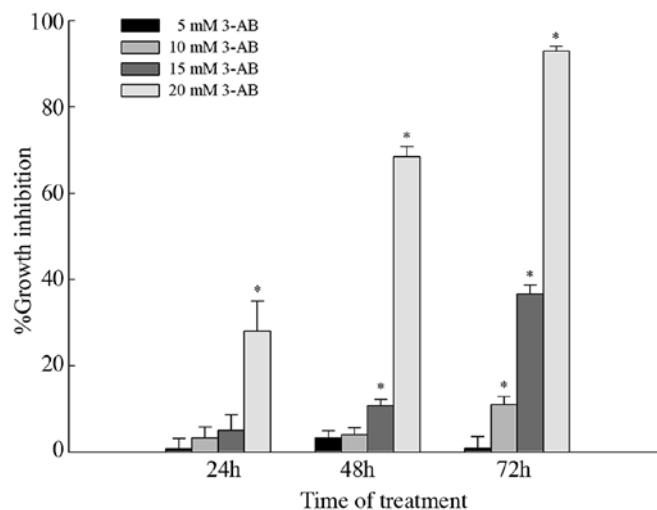


Figure 1. Growth inhibition of human osteosarcoma cells subjected to 3-AB. U2OS cells were treated with varying concentrations of 3-AB, the inhibition rates were reported as % of control. Data are representative of three independent experiments. *P<0.05, statistically different as compared with the control group.

Technologies Inc.), we followed the protocols to process the cells, and measured the absorbance at 405 nm.

Western blot analyses. Cells were washed with ice-cold PBS and then collected with 0.05% trypsin, collected cells were lysed on ice with lysis buffer, after centrifugation (12000 \times g for 10 min, 4°C), the supernatants were collected and stored at -80°C as whole cell extracts. Equal amounts of protein (~60 μ g) were run on SDS-page, and transferred electrophoretically to PVDF membrane. The membranes were blocked for 1 h at room temperature with 5% skim milk in PBST, then incubated with various primary antibodies at 4°C overnight. After washing with PBST for 30 min, the membranes were incubated for 1 h with horseradish peroxidase-conjugated secondary antibody. Bound antibody was detected using the ECL Western blotting reagent with chemiluminescence and detected using Fuji Medical X-ray film.

Migration assay. The migration assays were performed in a 6.5-mm Transwell chamber with 8.0- μ m pore polycarbonate membrane insert (Corning), the inserts coated with Matrigel Basement Membrane Matrix (BD Biosciences) were placed over the bottom chambers, serum-containing medium (10% FBS) was used as chemo-attractant and serum-free medium containing 0.1% BSA as negative control. U2OS cells were divided into two groups, one group served as a control, while the second group was pretreated with 10 mM 3-AB for 24 h, 30,000 cells were suspended in 100 μ l in serum-free medium containing 0.1% BSA and added to the upper chamber. After 24-h of incubation at 37°C, the non-migrated cells on the upper surface of the filter were removed by gentle swabbing with cotton-tipped applicators. The cells that had migrated to the lower side of the chamber were fixed with 4% paraformaldehyde for 15 min at room temperature. After complete drying, the cells were stained with 0.1% crystal violet. Migrated cells were scored in 8 random fields using \times 10 objectives.

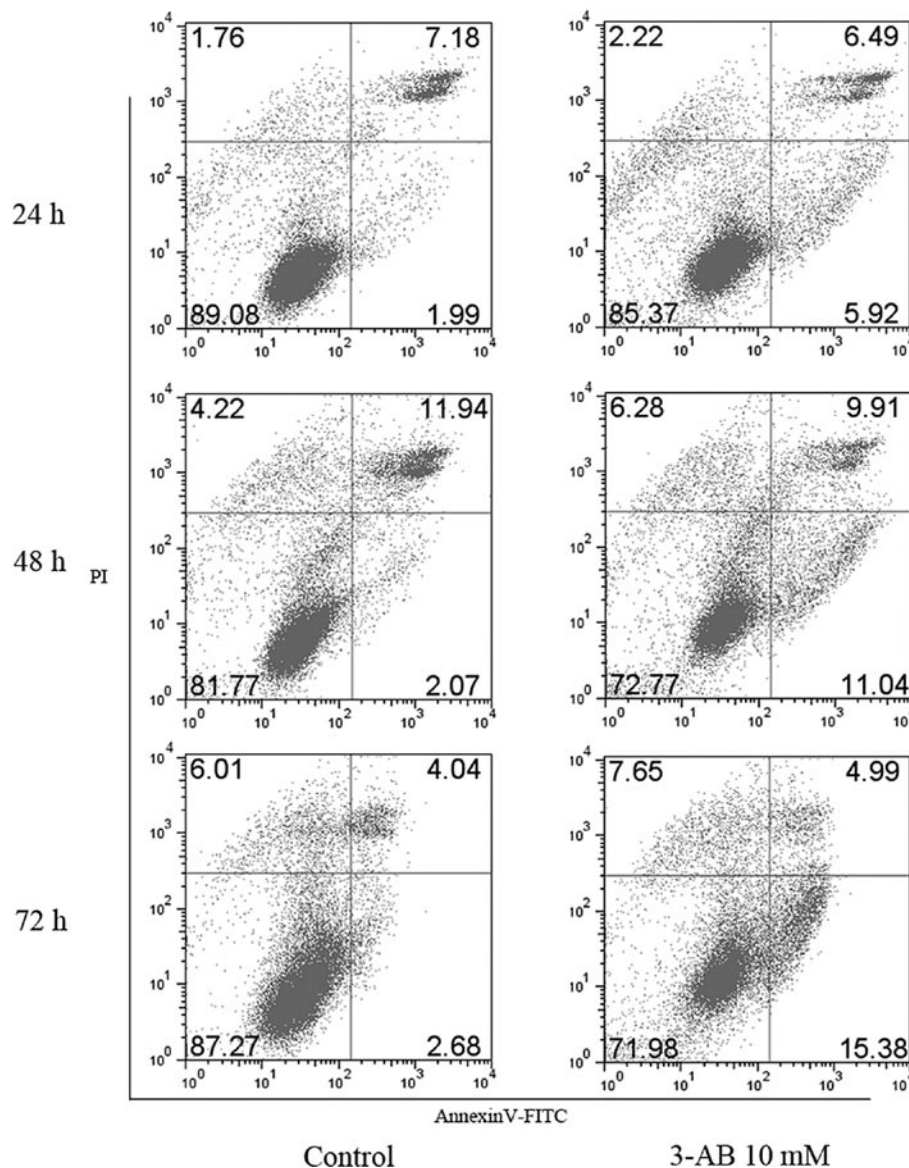


Figure 2. Assessment of apoptosis of U2OS cells by flow cytometry. U2OS cells were treated with or without 10 mM 3-AB for 24, 48, and 72 h, the 3-AB-treated groups exhibit a higher apoptosis ratio than the control groups.

Cell cycle analyses. Cells were suspended twice with ice-cold PBS. The cell densities were adjusted to $1-2 \times 10^6$ cells/ml in each tube. The chilled 80% methanol was added to each tube and stored at -20°C overnight. The tubes were centrifuged and the methanol was removed. Cells were suspended in 200 μl PBS. PI was added (10 μl) (5 mg propidium iodide, 0.1 ml Triton X-100, 3.7 mg EDTA in 100 ml PBS) and 10 μl of RNase solution (100 U/ml) and mixed gently and incubated in a dark at room temperature for 30 min. The samples were subjected to a flow cytometry for analysis within 1 h.

Results

3-AB inhibits the growth of osteosarcoma cells. Time- and dose-course experiments were performed after 3-AB addition to cell culture medium. Fig. 1 shows the effects of 3-AB on U2OS cells. Low concentrations of 3-AB (5 mM) showed no significant inhibition of cell viability. At concentrations

of 10 mM, reduction of cell viability in $\sim 10\%$ of cells took place after 72 h exposure. After treatment with 15 mM 3-AB the effects appeared to be significant at 48 h when a 10% inhibition was observed, while at 72 h a 36% inhibition was measured. When treated with 20 mM 3-AB cell viability was reduced by 28% within 24 h, by 68% after 48 h and by $\sim 93\%$ after 72 h.

The suppressive effects of 3-AB are associated with increased cell apoptosis. To explore the potential mechanism by which 3-AB suppresses osteosarcoma cell growth, cell apoptosis characteristics of the 3-AB-treated and untreated cells were investigated. Flow cytometry analysis showed that the apoptosis indices of 3-AB-treated U2OS cells at 24, 48 and 72 h of culture were higher than those untreated cells at the same time points of culture (Fig. 2). Other classic markers of apoptosis such as caspase-3, Bax and Bcl-2 were analyzed. Caspase-3 is a cysteine protease known to take part in the execution phase

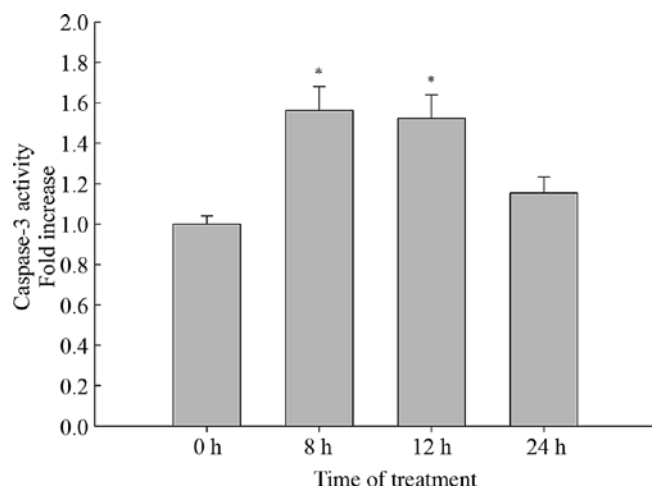


Figure 3. Caspase-3 activity of U2OS cells was analyzed by caspase-3 colorimetric assay. After treated with 10 mM 3-AB, the caspase-3 activity started to increase at 8 h; at 24 h the activity decreased. * $P < 0.05$, statistically different as compared with the control group.

of apoptosis, our findings (Fig. 3) showed that the activity of caspase-3 in U2OS cells started to increase after 8 h treatment with 10 mM 3-AB and at 12 h the caspase-3 activity was significant higher than the control, at 24 h the activity decreased, approximately equal to the control group.

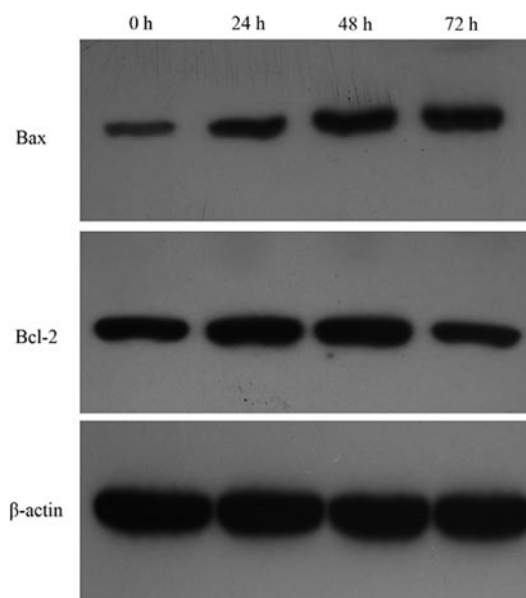


Figure 4. Analysis of Bcl-2 and Bax in U2OS cells by Western blotting. U2OS cells were subjected to 10 mM 3-AB for 24-72 h, the expression of Bax increased gradually, and the expression of Bcl-2 first increased and then decreased to initial level.

It is known that Bax and Bcl-2 are involved in apoptosis, with Bcl-2 decreasing and Bax increasing during the event. Western blot analyses (Fig. 4) showed that in U2OS cells Bax gradually increased after treated with 3-AB for 24, 48 and

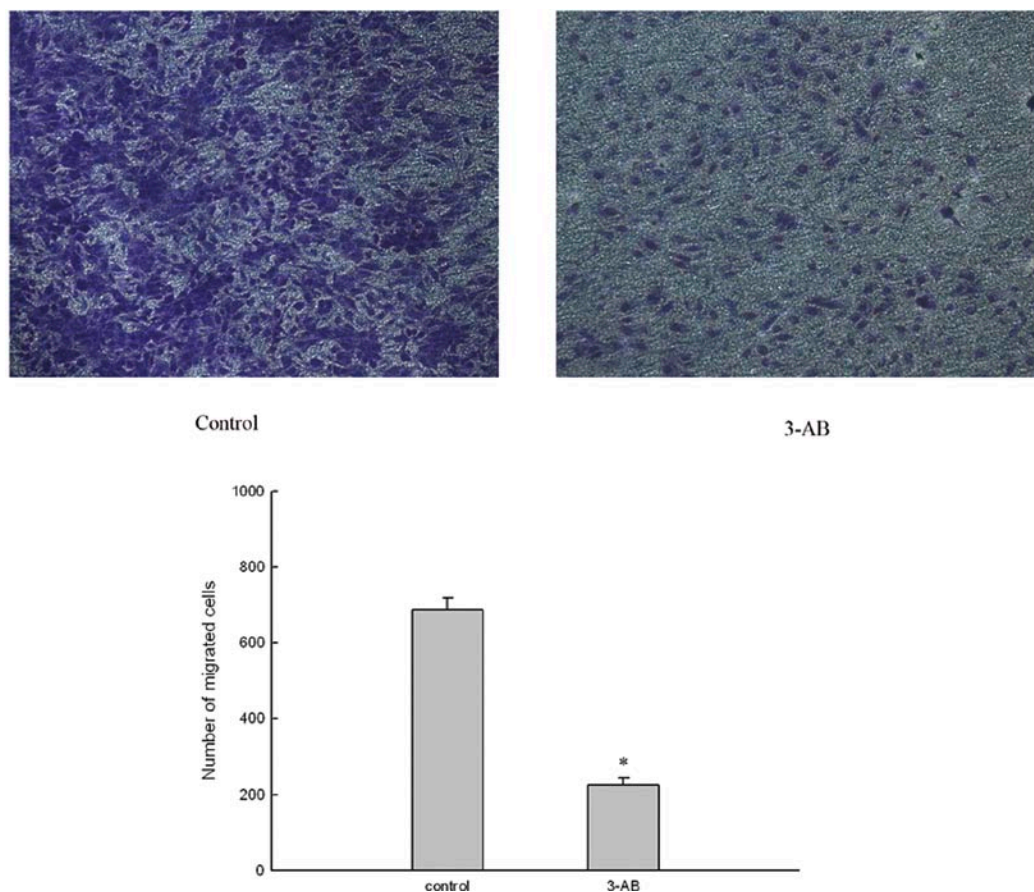


Figure 5. Cell invasion was analyzed by *in vitro* migration assay performed in transwell chamber. U2OS cells were treated as described in Materials and methods, the cell invasion were reduced after treated with 3-AB. Magnification, $\times 100$. * $P < 0.01$ statistically different as compared with the control group.

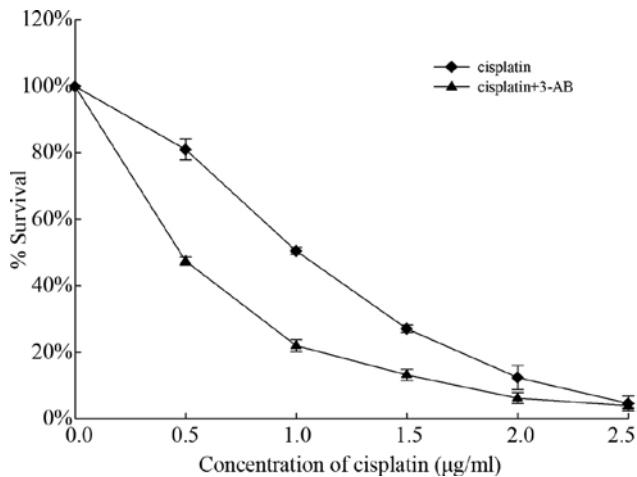


Figure 6. Results of cytotoxicity assays of cisplatin co-treated with 3-AB in U2OS cells. U2OS cells were treated with cisplatin alone or co-treated with 3-AB. The suppressive effects of cisplatin were enhanced by 3-AB.

72 h, but Bcl-2 seemed to increased after 24 and 48 h of treatment. At longer times of 3-AB treatment (72 h) Bcl-2 returned to initial levels.

3-AB attenuates osteosarcoma cell migration. The ability of cell chemotaxis was analyzed in an *in vitro* migration assay performed in transwell chamber. The results, illustrated in Fig. 5, show that 3-AB inhibited the migration of U2OS cells.

3-AB enhances the suppressive effects of cisplatin in osteosarcoma cells. The growth inhibition of 3-AB was investigated above, according to the results the maximum tolerated dose, the highest concentration at which at least 90% of the cells survive, was determined (15 mM 3-AB for 48 h), U2OS cells were co-treated with the maximum tolerated dose of 3-AB and varying concentrations of cisplatin. The result of CCK-8 assays are presented in Fig. 6 and the IC₅₀ values for cells treated with cisplatin alone and co-treated with 3-AB was calculated; our

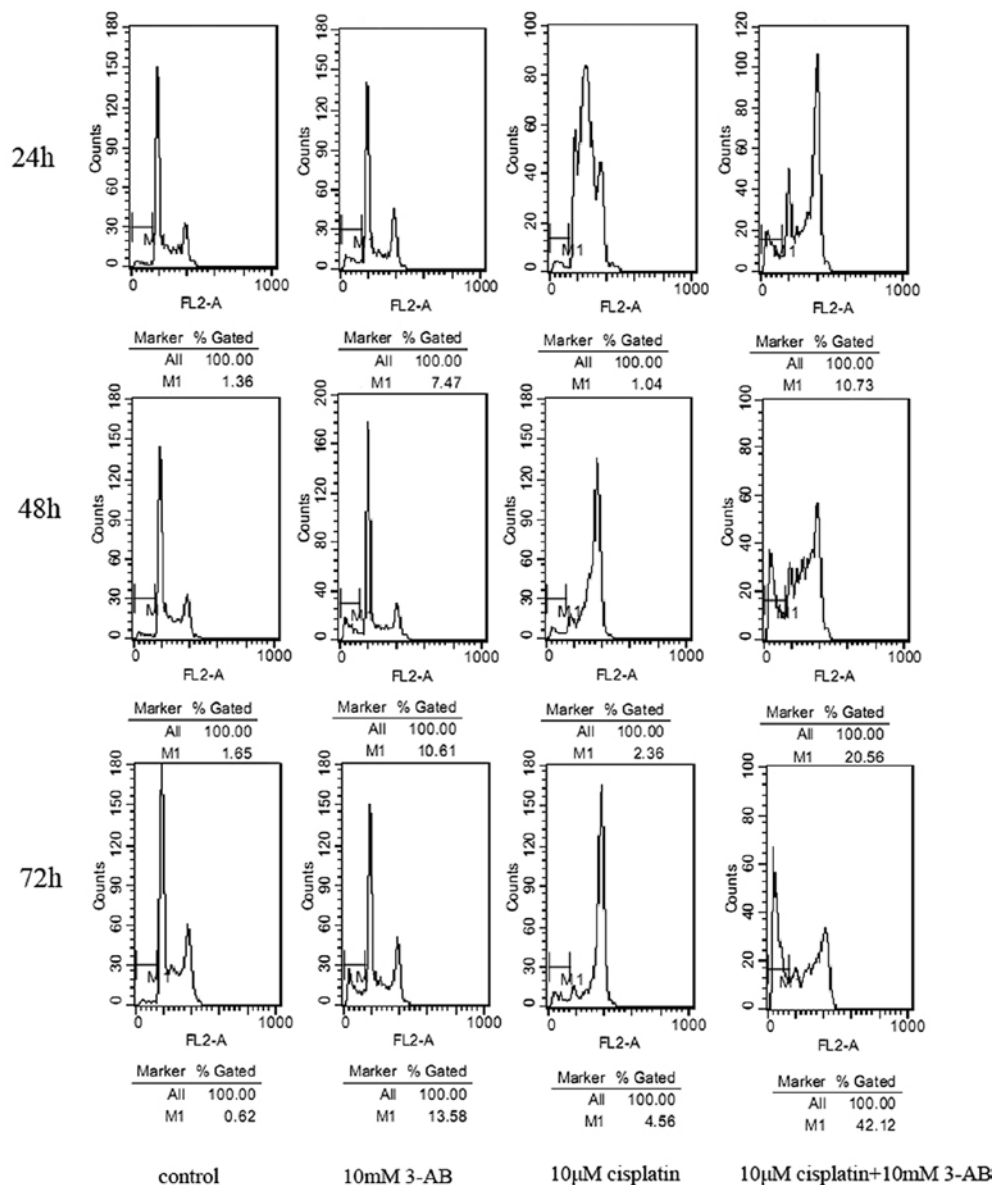


Figure 7. Assessment of apoptosis of U2OS cells subjected to cisplatin and 3-AB single and combined treatments for 24-72 h. Cisplatin plus 3-AB-treated groups exhibit a higher apoptosis ratio than the control groups and single agent-treated groups.

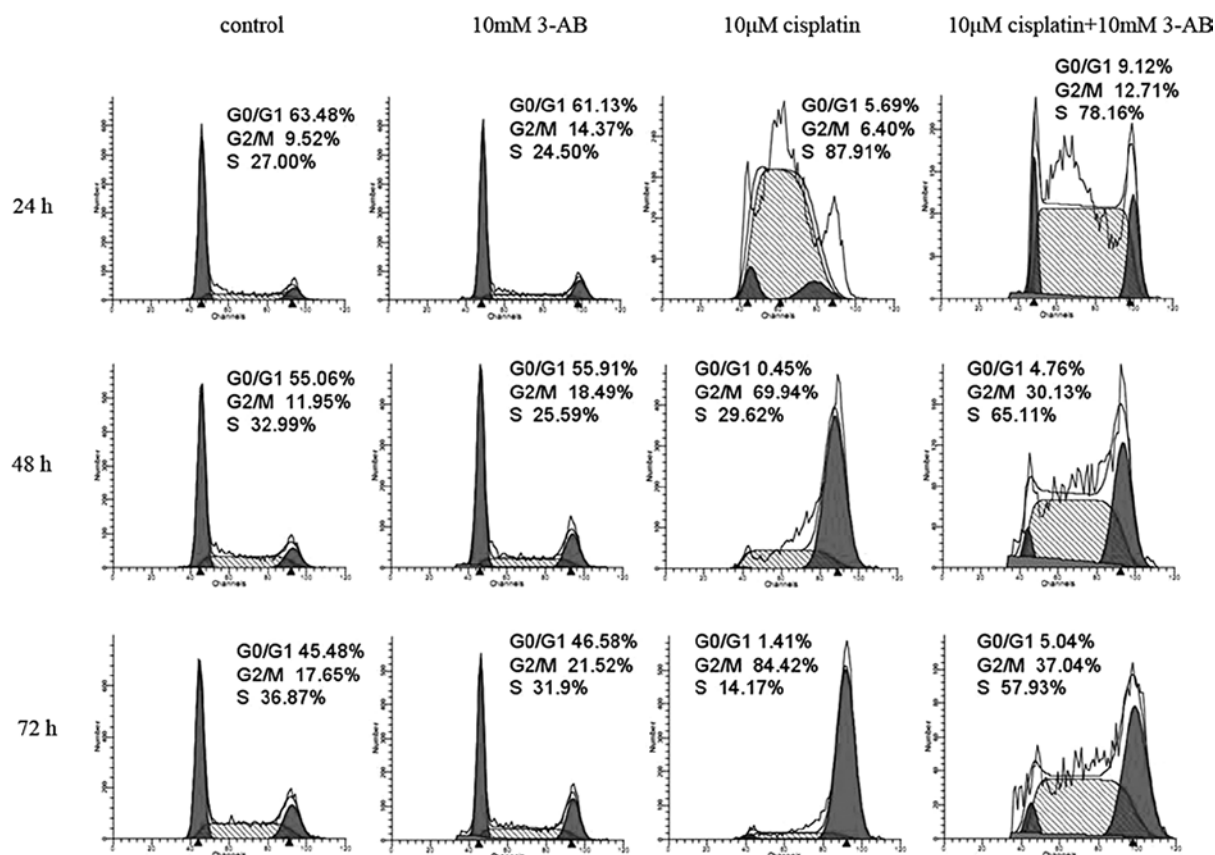


Figure 8. Cell cycle progression in U2OS cells subjected to cisplatin and 3-AB single and combined treatments for 24-72 h. The percentage of cells in the different phases of the cell cycle is indicated.

finding suggested that 3-AB sensitized U2OS to cisplatin by factor of 1.76.

Thereafter, we evaluated the effect of 6 h treatment with 10 μ M cisplatin in presence of 10 mM 3-AB on U2OS cell apoptosis, as shown in Fig. 7, compared with the control group, the group treated with cisplatin or 3-AB alone for 24, 48, 72 h had an increased apoptosis index, and when cisplatin was combined with 3-AB, the apoptosis index was higher than that with cisplatin or 3-AB alone.

Moreover, we analyzed the degree of cell cycle perturbation in U2OS cells at different times after either single drug or 3-AB and cisplatin-combined treatments. As shown in Fig. 8, compared to control, cell cycle kinetics was unaffected by 10 mM 3-AB alone, while 10 μ M cisplatin induced S phase accumulation, and then convert to G₂/M accumulation, interestingly, 10 mM 3-AB + 10 μ M cisplatin induced a more sustained S phase arrest.

Discussion

Since the resistance of cancer cells to DNA damaging agents stems from the modulation of DNA repair pathways, pharmacological inhibition of these pathways has been emerging as an effective tool for cancer treatment (7). PARP-1 is a molecular sensor of DNA breaks and it has a key role in the spatial and temporal organization of their repair, it regulates chromatin structure and DNA metabolism (25). Previous studies have indicated that pharmacological inhibition of PARP-1 results

in a delay in tumor formation and in a dramatic reduction in tumor size and multiplicity during chemically-induced skin carcinogenesis (26). Also PARP-1 inhibitor was able to suppress cell growth of liver cancer cell lines *in vitro* and HepG2-derived tumor development *in vivo* (27).

Our study suggested that 3-AB alone was able to inhibit the growth of the U2OS osteosarcoma cell line, and the suppressive effects were observed in a dose- and time-dependent manner. Further studies indicated that after treatment with 3-AB, the apoptosis rates increased. Then we tested the caspase-3 activity, the increase of caspase-3 activity after treatment with 3-AB was observed, which is in accordance with the increased apoptosis rates. Then we tested the expression of Bax and Bcl-2, Western blot analyses show that the expression of Bax increased and Bcl-2 first increased and then decreased to initial level. It is known that the Bcl-2 family plays a central role within the apoptosis pathway. This family possesses both pro-apoptosis and anti-apoptosis molecules and the ratio of anti- to pro-apoptosis molecules such as Bcl-2/Bax has been assumed to determine the response to a death signal (28). Our results showed an increased ratio of Bcl-2/Bax after treatment with 3-AB, which is consistent with the increased apoptosis indices.

Some studies demonstrated that the mechanism of pharmacological inhibition of PARP-1 prevent cancer development include the selective killing of homologous recombination-deficient tumor cells, the down-regulation of tumor-related gene expression and a decrease in the apoptotic threshold in the co-treatment with chemo- and radiotherapy (15). Our

study suggested that the growth suppression of osteosarcoma cells mediated by the PARP-1 inhibitor 3-AB, was associated with the increased cell apoptosis.

We further studied whether 3-AB altered the motility of U2OS cells *in vitro*, our data showed that 3-AB significantly suppressed the cell invasion of U2OS cells, this suggested that 3-AB not only suppresses proliferation and induced apoptosis of U2OS cell but also inhibits its invasion.

Cisplatin is one of the most commonly prescribed chemotherapeutic drugs used to treat solid cancers including osteosarcoma. Platinum resistance, either intrinsic or acquired, is a major clinical problem. By inhibiting poly (ADP-ribose) polymerase-1, the chemo- sensitivity of cisplatin to tumor cells can be increased so that cisplatin resistance can be circumvented. For example, PARP-1 inhibitor 3-aminobenzamide (3-AB) increases the cytotoxic activity of the platinum compounds cisplatin, trans-[PtCl₂ (4-picoline) (piperazine)] and trans-platin against CH1cisR cisplatin-resistant ovarian tumor cells (29). The present work demonstrated that PARP-1 inhibitor 3-AB was able to sensitize the human osteosarcoma cell line U2OS to cisplatin, and the combination of 3-AB with cisplatin determined a higher apoptosis ratio and prolonged arrest of the cell cycle.

In conclusion, our findings demonstrated that the PARP-1 inhibitor, 3-AB, was able to suppress cell proliferation of U2OS cells and induce cell apoptosis. In addition, 3-AB suppressed the cell invasion and enhanced the suppressive effects of cisplatin in U2OS cells. This finding exerts a profound impact in the search for a more effective treatment for human osteosarcoma. Future studies will be focused on preclinical and clinical research of the PARP-1 inhibitor for the treatment of human osteosarcoma.

Acknowledgements

This research was partially supported by the National Natural Science Foundation of China (no. 51077065) to Dr Hua Wu. We thank Yuan Li, Shuang Wei, Daqian Zhan for their excellent technical assistance.

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